# Embryonic asymmetry: **The left side gets all the best genes**

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**The origin of left–right developmental asymmetry is a continuing puzzle, but some recent results provide new insights into the steps leading to organ asymmetry – implicating the homeobox protein Pitz-2 in one key step – and others support a model of symmetry-breaking that involves the chirality of microtubules.**

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Gastrulating chordate embryos appear bilaterally symmetrical about a midline plane, but the internal body plans that emerge are always asymmetric, and the differences between left and right sides are consistent in all normal individuals of a species. The earliest and most profound asymmetries develop within the heart, the rightward looping of the future ventricular tube being the most obvious. As the heart develops, the left and right atria adopt distinct morphologies, eventually yielding the complex, four-chambered organ seen in the adult. Elsewhere in the viscera, asymmetries become apparent — the lungs and liver have different left and right lobe morphologies, the stomach and the spleen lie to the left, and so on. Indeed, the whole embryo 'turns' to one side during development.

Abnormal left–right development can give a strict mirror-image of the usual body plan, known as *situs inversus*, where morphologically left and right structures — such as atria, ventricles, lung buds and so on — lie on the actual right and left, respectively, of the embryo. The left and right sides can be independently assigned to individual organs. Both sides of a paired structure, such as the lungs, may even adopt the same lateral identity — for example, they may both have the typical left-side morphology, which is known as left isomerism.

The developmental mechanisms that normally provide this consistent handedness are often discussed as specifying the 'left–right axis', but this is somewhat misleading. Unlike the other axes, handed laterality is quantal, rather than graded; plus, it is meaningless without reference to the other axes. Theory predicts that breaking bilateral symmetry is likely to involve a (macro)molecule that is chiral, and that its handedness must be converted to a one-sided signal, which can be transmitted, and ultimately interpreted in the asymmetric morphogenesis of the heart, gut, and so on [1]. This, in turn, suggests that breaking symmetry probably involves unique developmental components, whereas the downstream transmission and interpretation of asymmetry could use the usual tool-box of molecular signals and receptors.

The first insights into the asymmetry transmission pathway came from screening likely signalling molecules for asymmetric expression patterns in the chick. This approach identified a pathway leading from activin signalling in the primitive streak and node — the earliest axial organisers of the embryo — through left-sided node expression of *Sonic hedgehog* (*Shh*), and culminating in expression in the left lateral plate mesoderm of *nodal*, which encodes a signalling molecule of the transforming growth factor β (TGF-β) family. Although earlier events may not be well conserved, similar left-sided *nodal* expression is seen in birds, mammals and amphibia (reviewed in [2]). In mice, two further genes encoding TGF-β family members with asymmetric expression patterns have been identified: *lefty-1*, which is expressed in the left half of the ventral neural tube — the prospective floorplate — and the closely related *lefty-2*, which is expressed in a domain almost identical to that of *nodal* [3].

A flurry of recent papers have now reported that the Bicoid-related homeobox transcription factor Pitx-2 — also known as REIG, Otix-1, Brx-1 or Ptx-2 — acts immediately downstream of Nodal and/or Lefty, and appears to drive asymmetric morphogenesis [4–8]. In mouse, chick and *Xenopus*, *Pitx-2* was found to be expressed in an asymmetric pattern initially almost identical to that of *nodal* (and mouse *lefty-2*) in the left lateral plate. *Pitx-2* expression was seen to last longer, however, and to spread into the left side of the developing heart, gut, lungs and body wall. *Pitx-2* has been convincingly positioned downstream of the *Shh–nodal* pathway, both by ectopic expression studies, and by signal-blocking experiments in chick and *Xenopus* embryos. In the mouse, several mutants in which the development of left–right asymmetry is disrupted — *lefty-1*$^{-/-}$, *inversus viscerum* (*iv*) and *inversion of embryonic turning* (*inv*) — have been observed to affect *nodal* and *lefty-2* expression in the lateral plate, and *Pitx-2* expression appears to follow in step.

There is similarly convincing evidence that *Pitx-2* has a role in specifying 'leftness' at the organ level. Ectopic expression of *Pitx-2* in chick, using a retrovirus vector, was found to disturb the asymmetric morphology of the heart and gut, and also the normal orientation of 'embryo turning'. Within the heart, the most common result of

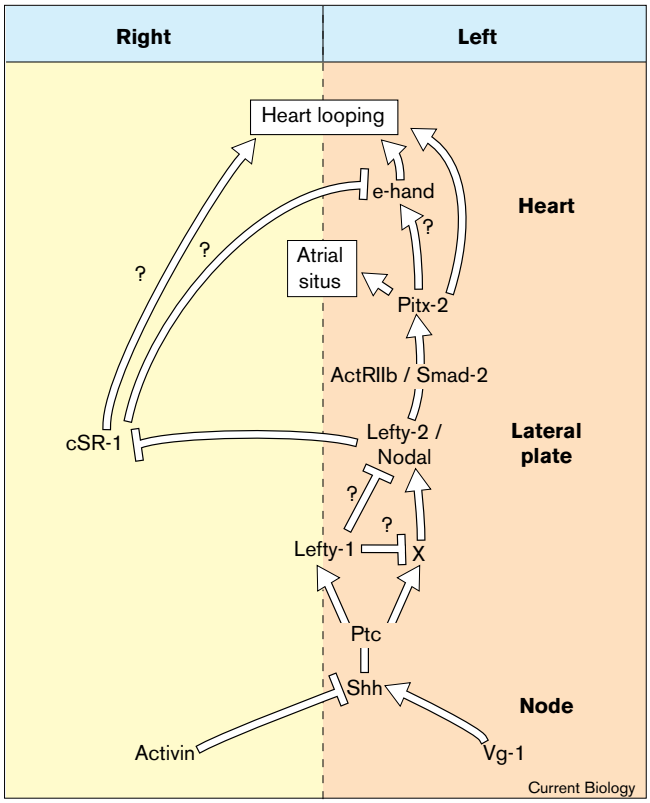
bilateral *Pitx-2* expression — induced by ectopic right-side expression — was development of a symmetrical ventral loop, though some inverted loops were also seen. When *Pitx-2* expression was both extinguished on the left and induced on the right, then an increased incidence of inverted loops was seen (in addition to the symmetrical loops also seen with bilateral *Pitx-2* expression) [5]. Furthermore, left atrial isomerism — where both atria develop with the typical left-sided morphology — is observed in the bilaterally *Pitx-2*-expressing *lefty-1* mutant mice [9]. Whilst failure of the developing heart to undergo looping can be a non-specific defect, the other observations suggest a direct link between *Pitx-2* and heart laterality, with bilateral *Pitx-2* expression causing left isomerism.

*Pitx-2* is thus involved in the most distal known step in the pathway that tells organs which side is on the left and how to develop in a left-like fashion. Given the conservation of *nodal* signalling, the conserved role of *Pitx-2* is perhaps unsurprising. The fact that many (perhaps all) developing asymmetric organs use the same lateral cue, despite their very different morphogenesis, shows that *Pitx-2* provides positional information, rather than driving a particular morphogenetic event. As expected, *Pitx-2* is also involved in other developmental pathways — it was first isolated by positional cloning as the gene responsible for Reiger syndrome, in which there are defect of the eyes, teeth, craniofacies and umbillicus [10]. There are no defects of laterality in Reiger syndrome, which is an autosomal dominant disorder, so one copy of *Pitx-2* may be sufficient to signal laterality even though it is not for other developmental pathways. This may reflect the quantal nature of left–right specification; no doubt the point will soon be resolved with the generation of *Pitx-2* knockout mice.

The downstream effectors of asymmetric morphogenesis have not yet been identified, though in the case of the heart there are a number of promising candidates. One is the mouse gene *e-hand*, which encodes a helix–loop–helix transcription factor and is required for heart looping [11]. Expression of *e-hand* is transiently upregulated — apparently with the help of the symmetrically-expressed homeobox transcription factor nkx2.5 — in the left side of the linear heart tube just before looping [12]. Another TGF-β-like molecule, bone morphogenic protein 4 (BMP-4), is also expressed more strongly on the left of the zebrafish heart tube before looping [13], but this is not observed in other species.

The activin type IIb receptor (ActRIIb) seems a good candidate for being the receptor that detects Lefty/Nodal signals [14], possibly signalling to the nucleus via activation of Smad-2 [15]. Mutations affecting these proteins result in a high incidence of right isomerism, which fits with the idea that they block a left-sided signal. A pathway thus emerges flowing from Nodal/Lefty-2 via

**Figure 1**




The diagram shows proposed interactions leading to asymmetric left–right development in vertebrates. A role for Vg-1 has been shown only in *Xenopus*; roles for activins, Shh, Ptc and cSR-1 have been demonstrated only in the chick; and roles for Lefty-1, Lefty-2, the activin type IIb receptor (ActRIIb) and Smad-2 have been demonstrated only in the mouse. Only the heart is shown, but Pitx-2 is involved in directing other visceral asymmetries. Arrows represent inductive interactions, bars repression and question marks putative links for which there is no direct evidence at present.

ActRIIb and Smad-2 to upregulation of *Pitx-2* (Figure 1). Although this pathway certainly appears to govern atrial laterality, it appears that, in the mouse, it does not dictate the direction of heart looping. In *lefty-1*$^{-/-}$, *ActRIIb*$^{-/-}$ and *Smad-2*$^{+/-}$ *nodal*$^{+/-}$ mice, in all of which *Pitx-2* expression is abnormal, the normal laterality of atrial morphology is affected, but the direction of heart looping is normal. Heart looping is, however, affected by ectopic *Pitx-2* expression in chick embryos. This perhaps rather surprising species difference correlates with the more extensive *Pitx-2* expression observed in the hearts of chick embryos compared with those of the mouse.

The symmetrical hearts generated by ectopic bilateral *Pitx-2* (or *nodal*) expression in the chick are, however, different from those generated by bilateral *Shh* expression. The latter hearts, like those of *iv* mutant mice, loop, but in either direction [16]. This implies the involvement of

additional factors, also under the control of *Shh* in the chick, in determining the direction of heart looping. The chick transcription factor Snail Related-1 (cSR-1) might have a role here — *cSR-1* is expressed in the right lateral plate mesoderm, affects the direction of looping and is also modulated by Shh [17].

In species other than the chick, where Shh is strongly implicated and thought to act via a predicted 'gene *X*' in the paraxial mesoderm [18], the inducers of *nodal* and *lefty* have not been identified yet. The involvement of Shh and activins is ruled out in mice by the lack of any laterality defects caused by mutations in their genes, but the node, however, remains the likely conduit for the signalling process that induces left-side *nodal* and *lefty* expression. *Brachyury* mice [19] and HNF3-β mutants [20] — rescued from primitive streak defects by chimerism — have severely abnormal nodes, and both almost completely fail to express *nodal* or *lefty-2* in the lateral plate (although *lefty-2* expression is occasionally seen in the lateral plate of HNF3-β mutants). A further piece of evidence implicating the node is that it is the site of expression of 'left–right' dynein, as explained below.

The midline emerging anterior to the node, the developmental forerunner of the spinal cord, may also provide such signalling. But perhaps the most attractive model to come out of a rather confusing collection of data gives the midline a barrier function. This model suggests that some key secreted component of the signalling pathway — such as the product of *left-2* or *nodal*, or perhaps of their predicted inducer 'gene *X*' — is prevented from diffusing to the right by the action of Lefty-1 in the midline. Lefty-1 might act as a barrier, either by direct inactivation of the secreted signalling molecule, or by blocking signalling. The model has the benefit of explaining the rather puzzling bilateral expression of *lefty-2* and *nodal* seen in *lefty1*$^{-/-}$ mice.

Support for the idea that Lefty proteins might block TGF-β signalling comes from their structure — they lack a cysteine residue that is normally present in TGF-β family proteins, where it is required for dimerisation and thus receptor activation — and from their apparent antagonism of BMP-4 action [3]. A more direct action is suggested, however, by the ability of mouse Lefty protein to inhibit later expression of *nodal* and *Pitx-2* when applied to the early chick node [7], and by the bilateral upregulation of *nodal* and *Pitx-2* expression that was found to follow application of a dominant-negative receptor construct predicted to bind all members of the subclass of TGF-β family ligands that includes Lefty. So, if the chick also has *lefty* genes, endogenous Lefty-1 might inhibit expression of *nodal* and *lefty-2*, thereby keeping their expression domain confined to the left lateral plate. The dominant-negative receptor might block endogenous Lefty-1 signalling, thus relieving this inhibition.

Although appealing, not all the evidence fits nicely with this model. The upregulation of *lefty-2* expression in floorplate observed in *lefty1*$^{-/-}$ mice would, considering the gene's extreme similarity to *lefty-1*, be predicted to perform the barrier role — so why does the barrier not function in these mutant mice? Perhaps a slight temporal delay in *lefty-2* upregulation is sufficient to allow leakage of the signal to the right. Another puzzle is why *lefty-1* is expressed specifically in left prospective floorplate if its product simply acts a barrier — why not throughout the whole prospective floorplate? The barrier model would also predict that one would see bilateral *nodal* and *left-2* expression in the mouse *no turning* mutant, in which heart looping is random and *lefty-1* expression is absent from the midline [21]. But, in fact, right-sided, left-sided and a complete absence of expression of these genes are also observed.

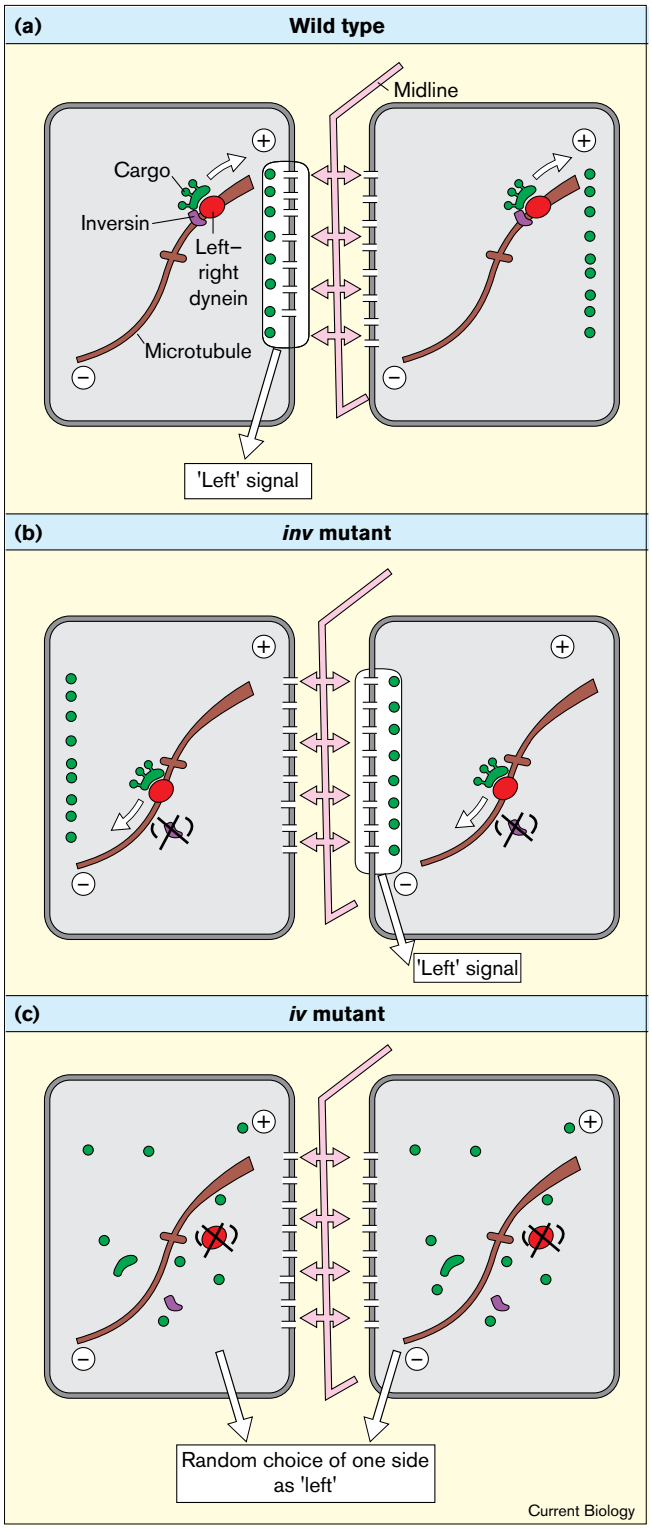
So much for the relays of information, but what about the initial breaking of symmetry? Mammalian mutants are providing new clues about this intriguing process, in which we might expect to see unique components playing a role. The gene *ZIC3*, which encodes a transcription factor related to the product of a *Drosophila* pair-rule segmentation gene, is mutated in some human laterality syndromes [22]. *ZIC3* expression in the mouse primitive streak suggests an early action, but no function is yet known. The suggestion that mutations of the gap junction protein connexin 43 are also associated with human laterality syndromes has not been confirmed, but in *Xenopus*, disruption of gap junctions at the blastocyst stage has been found to cause laterality defects [2]. The most interesting results, however, are undoubtedly those that have come from the cloning of the mouse genes *iv*, mutations in which cause randomised laterality, and *inv*, mutations in which cause a uniform inversion of laterality.

The *iv* gene encodes a dynein heavy chain, called left–right dynein, and is expressed in the mouse node [23]. Dyneins are motor proteins that come in various forms; best known are the axonemal dyneins, which power the beating of cilia, and cytoplasmic dyneins, which play a role in intracellular transport. Left–right dynein closely resembles the axonemal dyneins, but it is found in both ciliated and non-ciliated cells. Although cilia defects have long been known to be associated with human laterality syndromes, *iv* mice show no obvious cilia defects. But both an absence of cilia and laterality defects are observed in mice lacking the transcription factor HFH-4 [24]. It seems that HFH-4 controls the expression or action of microtubules, or associated components, required both for assembly of cilia and for left–right specification.

The *inv* mutation results in the almost complete loss of expression of *inversin*, which encodes a large protein with ankyrin repeats and is normally expressed at low levels throughout the gastrulating embryo [25,26]. Although

**Figure 2**

A model for symmetry-breaking, based on the model in [1]. **(a)** Cells are polarised relative to the midline (pink plane and arrows), so that a component is present on their medial face only (pink bars). Microtubules (brown) are aligned in cells relative to the anteroposterior and dorsoventral axes. A complex of left–right dynein (red), inversin (purple) and other components (green) normally moves towards the plus end of the microtuble. This results in accumulation of some putative determinant transported by the complex at the lateral face of cells on the right of the midline, but at the medial face of left hand cells, where there can be an interaction with the medial component, triggering the asymmetric cascade. **(b)** The absence of inversin, as in *inv* mutants, is postulated to cause the dynein motor to reverse direction, and thus the interaction occurs on the right and laterality is inverted. **(c)** The absence of left–right dynein, as in *iv* mutants, prevents transport, so interaction could occur on either side, leading to randomization of laterality.




ankyrin repeats are common mediators of protein–protein interactions, their arrangement in inversin is rather unusual, only having been seen previously in ankyrins themselves and in the *Caenorabditis elegans* protein Unc44, which specifies polarity in extending neurons. Intriguingly, ankyrins and Unc44 are both known to bind to both cytoskeletal and cell membrane components.

How might left–right dynein and inversin act in symmetry-breaking? We suggest this may involve the known directional movement of dyneins towards the minus end of microtubules. For some kinesin family motor proteins, it is known that factors acting outside of the motor domain can determine direction of movement [27]. Perhaps, then, left–right dynein and inversin normally interact in a microtubule motor complex, which loses all activity in the absence of left–right dynein, but reverses direction in the absence of inversin. This idea can be superimposed on a model proposed by Brown and Wolpert [1], with microtubules being the chiral reference 'F' molecule (Figure 2). This modified model accommodates the consistent inversion of *inv* mutants and the randomisation of *iv* mutants, but it is obviously simplistic and it goes no way to explaining the complex phenotypes of *iv* and *inv* mutants. It does, however, pose testable hypotheses about protein–protein interactions that might be involved in symmetry-breaking.

One well-known developmental phenomenon that might be relevant in this context is the cortical rotation triggered by fertilisation of a *Xenopus* egg. Disruption of this microtubule-driven process has been shown to result in abnormal left–right development. It has been proposed that this rotation might result in asymmetric distribution of active Vg-1, another TGF-β-related protein and one that has been shown capable of triggering the left sided signalling cascade in *Xenopus* [28]. Microtubules are also implicated in laterality determination in *C. elegans*, where handed orientation of the mitotic spindle in the earliest cell divisions appears to determine later morphological asymmetries [29]. So, the current picture is of symmetry-breaking by a chiral cytoskeleton, which sets up an asymmetric signal within the node. This leads via 'gene *X*' and *nodal/lefty-2* to *Pitx-2* expression on the left, being confined there by *lefty-1* action in the midline. *Pitx-2* is then responsible for directing the asymmetric morphogenesis of

the viscera. No doubt in a couple of years we will laugh at such naiveté.

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